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CERTAIN SAPROPHYTIC BACTERIA
AGAINST THE BACILLUS
TYPHOSUS GAFFKY

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITY OF WISCONSIN
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THE ANTAGONISM EXHIBITED BY CERTAIN SAPROPHYTIC BACTERIA AGAINST THE BACILLUS TYPHOSUS GAFFKY.*

WILLIAM DODGE FROST.

(From the Bacteriological Laboratories of the University of Wisconsin.)

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INTRODUCTION.

The effect which various agencies have on the life of Bacillus typhosus outside of the human body is a subject of great hygienic importance. Any information which will throw light on this subject is sure to receive due consideration.

It is held by some observers that this organism never finds conditions favorable for its development outside of the body, and that its existence in nature is measured by a few days, or at most a few weeks. Others, on the other hand, believe that this germ may lead a saprophytic existence, and that its life in nature may be prolonged indefinitely.

Whichever view may be ultimately shown to be correct, it is certainly true that the extracorporeal sojourn of the typhoid germ is influenced by certain factors, *e. g.*, the nature of the food substances, the amount of moisture, etc. The effect of these various factors has been repeatedly studied, and it may be presumed that, in a general way, their influence is fairly well understood.

There are, however, a number of factors whose influence is more or less uncertain. Of these perhaps none is more important or worthy of more careful consideration than the effect which various other bacteria have on the typhoid germ in their association with it.

Bacteria in nature occur almost invariably in mixed cultures. Their association may be without effect on the various species, or it may affect them in various ways. They may offer mutual or one-sided aid, and thus live in a symbiotic relation. They may, on the other hand, offer mutual or one-sided injury, *i. e.*, they may exert an antagonism on one another.

The present paper is a record of experiments performed for the purpose of determining the effect of the association of other bacteria on the typhoid germ. The bacteria studied are those which the typhoid germ would be likely to meet with in nature. All of them are well-known saprophytes.

Where an antagonism has been shown to be exerted an attempt has been made to determine the laws which govern this antagonism.

HISTORICAL REVIEW.

The fact that certain bacteria are antagonistic in their action toward B. typhosus has been known for many years.

v. Freudenreich[1] in 1888 determined that when certain bacteria were grown in flasks of broth for some time, and then filtered through a porcelain filter, the typhoid germ failed to grow in the filtrate in some cases, and in other filtrates it grew only feebly. The organisms which allowed the typhoid germ to grow feebly in its by-products were Staphylococcus pyogenes aureus, B. typhosus, Bacillus of chicken cholera, Spirillum of asiatic cholera, Spirillum of Miller, and Spirillum of Denecke. Those which did not permit the growth of the typhoid bacillus were: Staphylococcus pyogenes albus, Staphylococcus pyogenes fetidus, B. pyocyaneus, and B. phosphorescens; while in the case of Bacillus of symptomatic anthrax its growth was only delayed, and in the filtered cultures of Spirillum of Finkler and Prior it grew well.

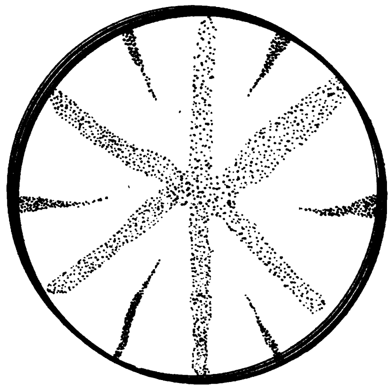


FIG. 1.—Drawing illustrating Garré's method of studying antagonism—alternate streaks. The large streaks of Ps. fluorescens were made and allowed to grow twenty-four hours, and then streaks of B. typhosus were made which grew only at the circumference of the plate.

Garré[2] in 1888 showed that Pseudomonas fluorescens putida produces in its growth on artificial media substances which are antagonistic to the typhoid germ. He grew this organism on gelatin, and then scraped it off and seeded the typhoid germ, and found that it would not grow, although Ps. fluorescens putida would grow on media from which a growth of B. typhosus had been scraped. He also demonstrated the existence of an antagonism by means of alternate streaks of the two organisms arranged radially on a gelatin plate. Where the streaks were near together the typhoid germ did not grow, but near the circumference, where the distance between the streaks was greater, both organisms developed normally (Fig. 1).

Olitzky[3] in 1891 worked on the antagonism which is exerted by B. fluorescens liquefaciens (Pseudomonas fluorescens), employing methods slightly modified from those of Garré.[2] This observer demonstrated a marked antagonism and laid stress on the hygienic importance of this fact.

Laws and Andrewes[4] in 1894, working on the duration of the life of B. typhosus in sewage, appeared to show that the presence in the sewage of B. fluorescens liquefaciens, and especially B. fluorescens stercoralis, shortened the life of the typhoid germ.

Sidney Martin[5] in 1898-1900, studying the growth of the typhoid bacillus in soil, determined that, while some of the soils furnished conditions favorable for the prolonged existence of the typhoid germ—in one case as long as 456 days—other soils presented conditions which were inimical to the growth

of the typhoid bacillus. This antagonism of certain soils he was able to trace to the effect of definite bacteria which he isolated and grew in pure culture. The identification of these bacteria was not established, but they were described as "Chichester 1, 2, 3, 4, and 5." The antagonism was tested by growing the organisms separately with the typhoid germ in 200 c.c. of sterile water to which 10 c.c. of sterile broth had been added. With No. 1 the typhoid bacillus died in less than twelve days at 8–12°, and six days at 37°. No. 5 killed out the typhoid germ in less than seven days at 37°. When these organisms were grown with the typhoid germ in sterile soil, No. 1 had gained the upper hand in twelve days at 8–12°, and in six days at 37°; No. 3, in fifteen days, and No. 5 in three days at 37°.

Rémy[6] in 1901 writes on the antagonism exhibited by B. coli for B. typhosus. He was, however, unable to show any antagonism, but maintained that the specific characters of both germs were changed. This change was in respect to their agglutination reaction as well as their cultural characters.

Horrocks[7] in 1901, working with Pseudomonas fluorescens, found that B. typhosus would not grow on gelatin which had already yielded a growth of Ps. fluorescens, but that gelatin which had served as a medium for B. typhosus would still permit the development of Ps. fluorescens. In sterile sewage he was unable to obtain B. typhosus after it had grown seven days with Ps. fluorescens. Working also on the effect on B. typhosus of its association with B. coli, with what he considered improved methods, namely, alkaline, glucose-litmus agar surface plates, he found that when these two organisms were grown together in sterile tap water B. typhosus could be isolated after twenty-one days, but in peptone water it could not be discovered after seven days.

METHODS AND MATERIALS USED.

METHODS USED.

A considerable number of methods have already been described by others for demonstrating the action which one organism may exert on another. It is proposed in this section briefly to describe these methods, and also, somewhat more in detail, the original methods which are here employed.

1. *Simultaneous culture on solid media.*– Garré was the first to use this method. As used by him, it consists of making streaks on the surface of agar or gelatin plates of the two organisms to be studied. The streaks are alternate, and may be either parallel, or radiating from a common center, or they may intersect at right angles. In all cases the streaks of the two organisms alternate. (Fig. 1 shows this method.)

In cases where the antagonistic substance does not diffuse for any considerable distance into the medium it is not satisfactory.

In this work the method has been modified in some instances so as to overcome this, as follows: The medium was seeded with B. typhosus, and when it had hardened, the opposing bacterium

was planted by making one or more streaks across the surface. (See Fig. 2.)

2. *Successive cultures on solid media* (*Garré.*)—Here one of the organisms is allowed to originate a good growth on a solid medium, as gelatin or agar. Then this growth is scraped off, and the second organism is sown on the medium. This is a useful method, and one that has not infrequently been used.

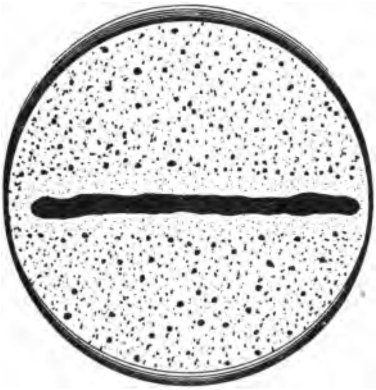


FIG. 2.—Drawing showing a modification of Garré's method. Agar plate heavily seeded with B. typhosus, and then streaked with B. vulgaris. Colonies are very small under and near the streak.

3. *Culture in filtered by-products.*—v. Freudenreich[1] grew one of the organisms to be studied in broth, and after some time filtered the medium through a porcelain Pasteur filter, and then seeded this sterile filtrate with the other organism, and by means of plate cultures, or otherwise, determined the behavior of this organism in the by-products of the other.

4. *Cultures on opposite sides of porcelain filter.*—Frankland and Ward[8] used a porcelain filter of the Pasteur-Chamberland system, partially filled with broth and arranged in a beaker of broth. (See Fig. 3.) The two germs to be experimented with are sown on opposite sides of the filter, where their behavior can be readily ascertained. Theoretically this method is ideal; practically, however, it is open to the objection that usually, if not invariably, motile bacteria will grow through the porcelain after a time. Indeed, a similar arrangement has been suggested for the separation of B. typhosus from the less motile B. coli. See in this connection Cambier,[9] and also von

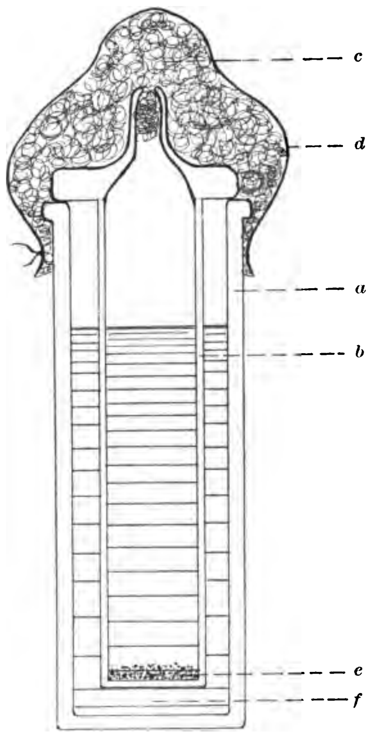


FIG. 3.—Frankland and Ward's method of studying antagonism. *a*, a glass jar; *b*, porcelain filter, Pasteur-Chamberland system; *c*, cotton wool; *d*, paper cap; *e* and *f*, broth in which are growing the two antibionts.

Esmarch[10] who gives figures showing the bacteria in the pores of the filter on their way through.

5. *Collodion sac method.*—Collodion sacs are formed as described in a former paper (Frost[11]). The tops are trimmed, and then they are drawn over test-tubes, from which the bottoms have been cut, fastened on with a thread or rubber band, and sealed with a little fresh collodion. The tube thus prepared is then partially filled with broth and placed in a small flask of broth. The test-tube with the collodion sac on the end is held in place in the flask by a packing of cotton. They are sterilized in the steamer or autoclave.

In this work the medium inside the sac has been seeded with B. typhosus, and the opposing germs have been placed in the flask. (See Fig. 4.) An arrangement similar to this was described by Ruffer and Crendiropoulo[12] in 1900. Results obtained by this method do not seem to have been published, and the arrangement described here had been in use for some months before their description was seen.

6. *Double-plate method* (*original*).—In this method a Petri dish is divided into two halves by means of a small glass tube or rod. After sterilization it is used by seeding an agar tube rather heavily with one of the organisms to be tested, and then pouring it into one-half of the plate. When this has hardened, a tube of sterile, uninoculated agar is poured into the other half. When this in turn is hardened, the other organism (in this work B. typhosus) is streaked across the surface of both halves, the seeded and the sterile. It is necessary, however, in order that the streak on both sides be equally heavily seeded, to streak each side separately. This is best done by having a loop bent at nearly right angles, and then with the charged loop to begin at the circumference and streak to the glass rod, sterilize and recharge the needle, and then continue the streak on the other side of the plate. In this way two parallel streaks are made across the plate. The streaks may be made as soon as the other organism has been planted, or as is usually desirable, the organism in the seeded half of the plate may be given an opportunity to develop before the other is streaked, thus making the antagonism more striking. (See Plate XVIII, Fig. 1.)

7. *Agar-block method* (*original*).—An agar jelly is made by dissolving 2 per cent. of agar-agar threads in distilled water, filtering through filter paper, and sterilizing. This agar is then

FIG. 4.—Collodion sac as arranged for the study of antagonism. The Bacillus typhosus is in the broth in the sac, while the opposing organisms are outside in the broth in the flask.

poured into a deep Petri dish or a crystallizing dish; the purpose being to obtain a cake of agar about an inch and a quarter in thickness. With a knife which has been sterilized in the flame the solidified agar is cut into blocks about two-fifths of an inch square and the height of the block. These blocks are seeded by stabbing them with a platinum needle charged with one of the organisms to be tested, in the same way that an ordinary stab culture is made. Care should be taken not to make the stab quite to the bottom of the block. The top of the block is sealed by touching it with a red-hot iron, *e. g.*, the head of a nail. In this way the bacteria which are left on the surface at the time of inoculation are killed, and when the agar cools the seal is complete. With a little practice blocks can be readily made so that their outer surface is perfectly sterile. These blocks are then placed by means of a pair of sterile forceps, in a broth test-tube culture of the other organism. When such blocks are placed in a culture medium, sufficient nutritive material diffuses through the layer of agar so that growth occurs along the line of puncture. In the case of sterile broth a heavy growth appears in the course of a few days. When these blocks are put in cultures, if any antagonistic factors are operative, growth may be only slight or not at all apparent. (See Fig. 5 and Plate XVIII, Fig. 2.)

MEDIA USED.

All the ordinary media used were made according to the procedures recommended by the Bacteriological Committee[13] and had a reaction of +1 (Fuller's scale). In a number of the experiments some of the differential media were used, *e. g.*, carbolized agar as used by Chick[14] and Hiss's[15] new media, but soon given up, as in the frequently crowded plates, and with cultures of B. typhosus which had been kept on artificial media for some time, there was no constant characteristic. All of the media were made from extract of beef and were sterilized in the autoclave at a temperature of 120°.

CULTURES OF BACILLUS TYPHOSUS USED.

Three cultures have been used. One, known in the laboratory as the M. H. culture, was received in 1898 from the United States Marine Hospital Service Laboratory, and since then has been used

in the laboratory as the standard culture, and was such when these researches were begun. Later, however, the organism began to show a tendency to clump in ordinary broth cultures, and it was then discarded for the second culture. This is designated as the P. D. 1. This was obtained in the fall of 1902 from Parke, Davis & Co., of Detroit, Mich. This gave a typical reaction with typhoid blood. A third culture has been used to some extent, P. D. 2. This was obtained from the same source about six months later.

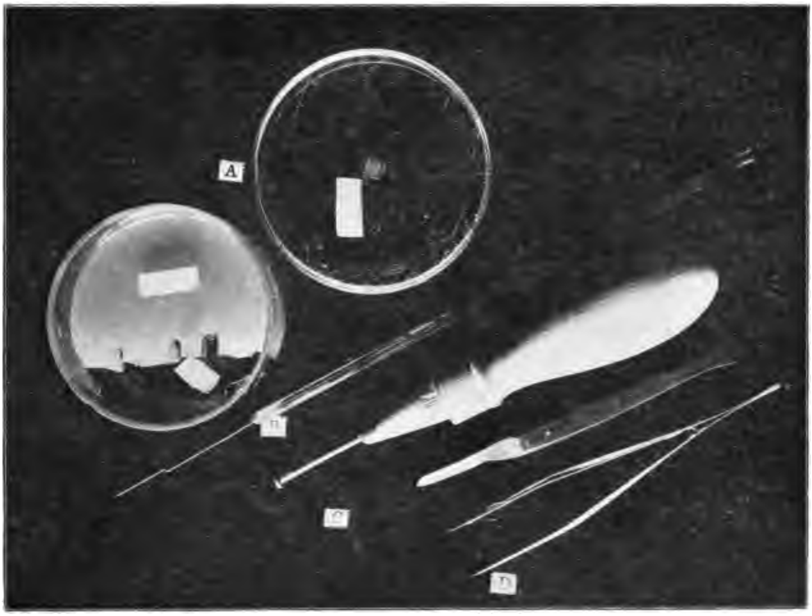


FIG. 5.—Method of making agar blocks. *A*, deep Petri dish and cover showing who blocks are cut out, also two on side; *B*, needle arranged to prevent making too deep stab; *C*, nail in handle for sealing end of block; *D*, forceps for handling blocks.

ANTAGONISM EXHIBITED BY VARIOUS MIXED CULTURES OF BACTERIA AS TESTED BY MEANS OF COLLODION SACS.

In studying the effect of the association of other bacteria on B. typhosus, one of the chief difficulties has always been the inability of recovering B. typhosus from such mixtures with ease and certainty. The use of selective media, especially those of Elsner and Hiss,[15] has given promise of success, but in my own

hands, at least, has proved to be of uncertain value, since the characteristic features of the typhoid on these media appear to become weakened in old laboratory cultures and are frequently closely simulated by other germs.

It would seem that ideal conditions would be reached in the study of the association of bacteria if one of the germs could be inclosed in a receptacle protecting it from intimate association with the other germs, but permitting the ready interchange of the nutritive solutions and by-products. Under these conditions the by-products produced by the germs growing on one side of the walls of the receptacle would presumably reach the germs on the other side, while they would at the same time be kept in pure culture and could be examined at will. With these conditions the effect of association ought to be as accurately determined as if the opposing bacteria were in actual contact, for at the present time we can conceive of the action of any germ exerted against another only as being accomplished by means of chemical poisons; and if the walls of the receptacle permit of a ready exchange of these, the conditions would be thoroughly satisfied.

The use of the collodion sac would seem to offer the desired means. It has been used so successfully in other work, especially with animals, that it seemed likely to be valuable in this connection.

METHODS.

The collodion sacs were made and arranged as previously described (see p. 604). The medium used outside the sac was ordinary broth, that within the sac was either broth or sterile tap water. The water used in this case was tap water drawn from Lake Mendota, Wis. This water has a rather high bacterial content. When used, the water was sterilized in the autoclave.

The medium outside of the sac was seeded with material containing, presumably, various kinds of bacteria, and B. typhosus was inoculated into the medium within the sac. In some instances the seeding of B. typhosus and the opposing germs was simultaneous; in other cases the seeding of B. typhosus was delayed until the bacteria outside of the sac had grown for some time. The start allowed the opposing germs varied from a few hours to

several days. In all cases the medium within the sac was seeded heavily with B. typhosus, for the reason that it seems certain, from a considerable series of observations in another connection, that surroundings which inhibit the growth, or actually kill, B. typhosus when it is present in small numbers are frequently inoperative when the seeding is heavy. In all cases in the following tables the figures refer to the number of germs present in a single loopful, the loop being one of the standard size recommended in the Procedures of the Bacteriological Committee.[13] The temperature at which the experiments were performed was that of the laboratory, 18–20° C., or in an incubator at 28° C. In the various experiments the temperature used is indicated.

The periods elapsing between the various quantitative determinations are not constant. Usually a number of determinations were made; in some cases they were few, but at the same time were considered sufficient for the purpose in hand.

SERIES I. SOIL BACTERIA.

This series was of a preliminary nature. It served to test the methods and suggested the succeeding work. The medium was broth and that in the flask was inoculated with a sample of soil obtained from the university campus. Flask No. 2 was not inoculated outside of the sac and served as a control. The culture of B. typhosus used was the United States Marine Hospital culture (M. H.). The temperature was that of the laboratory in summer, *i. e.*, about 22° C.

The data obtained are exhibited in the following table (Table I). It is also arranged graphically in Diagram I. The vertical distances, or ordinates, represent the number of typhoid germs per loop of medium. The horizontal distances, or abscissæ, represent the days. In regard to the meaning of the infinity sign used in the table see footnote to Table I.

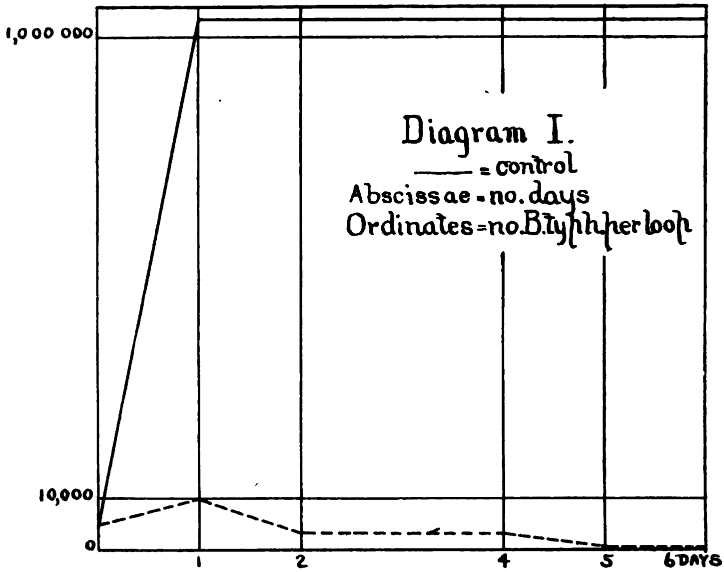
TABLE I.

EFFECT OF SOIL ORGANISMS ON B. TYPHOSUS.

No. of Experiment	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing on Agar Plates from One Loop of the Medium							Result
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 D.	4 D.	5 D.	6 D.		− Dec. + Inc.
1.......	Soil	M. H.	0	0	20–22°	5,290	9,600	3,300	2,900	200	100	..	− 98 %
2 (control)	....	M. H.	..	0	20–22°	4,900	∞*	∞	∞	∞	∞	..	+ ..

* The infinity sign (∞) means here that they could not be counted with a simple microscope. Later the compound microscope was used, and actual figures were given where the numbers were as great, possibly, as above.

The antagonism of these soil bacteria exerted against the typhoid germ is very strongly marked here; for while the experiments were not continued until the typhoid germ had entirely disappeared, it is to be noted that in the six days 98 per cent. of B. typhosus had been killed, and there is no doubt that in the course of a few days more they would have entirely disappeared. On the other hand, B. typhosus would live, as is well known, not only for many days, but for months, under conditions less favorable than those of the control culture.



SERIES II. SOIL BACTERIA.

This series was undertaken to confirm the results of the preceding experiment, and also to extend the observations on the extent of the distribution of the antagonistic organisms. The media outside of the sacs were inoculated with small amounts of soil from various sources which were selected with the idea of securing a variety of different micro-organisms.

Soil No. 1 was taken near the well at South Hall on the University campus. This well is frequently used, and the soil in the immediate neighborhood is kept constantly moist.

Soil No. 2 was obtained in the dust from the macadam road leading to Main Hall. This road is comparatively little used.

Soil No. 3 was taken from the woods north of Main Hall. This is a typical wood soil. It is a virgin soil.

Soil No. 4 was sand brought directly from a pit, and was being used to make a cement walk.

It will thus be seen that, while the soils all differ very materially, they are all to be classed as uncontaminated soils, except, possibly, sample No. 2, and probably not fitted to furnish conditions favorable for the development of B. typhosus. With the exception of sample No. 1, they are all dry soils.

The broth was inoculated, and the cultures were grown for six days, when it was intended to inoculate the sacs with B. typhosus. It was found, however, at this time that all of the sacs were imperfect and had become contaminated. They were then removed and replaced with sacs which had been tested by growing B. typhosus in them for twenty-four hours. It thus occurred that the soil bacteria had had opportunity for abundant development before B. typhosus was brought in contact with them. But it should also be noted that the sacs were very thickly seeded, and thus B. typhosus would be less easily affected. The temperature of this experiment was 28° and the typhoid culture the M. H.

The results are shown in Table II:

TABLE II.

EFFECT OF SOIL ORGANISMS ON B. TYPHOSUS.

No. of Experiment	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing on Agar Plates from One Loop of the Medium							Result
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	3 D.	5 D.				−Dec. +Inc.
1.......	Soil 1	M. H.	6 days	1 day	28°	∞*	∞	10	25	..	..	..	−100 %
2.......	" 2	"	6 "	1 "	28°	1,100	∞	200	10	..	..	..	−100 %
3.......	" 3	"	6 "	1 "	28°	∞	∞	∞	6	..	..	..	−100 %
4.......	" 4	"	6 "	1 "	28°	∞	∞	10	25	..	..	..	−100 %
No	con	trol											

The antagonism exerted here is more marked even than in the preceding series, being practically 100 per cent. decrease in five days. This result is obtained in spite of the fact that the sacs were very heavily seeded to start with. It is to be noted here also that the infinity sign has a special meaning, as explained in footnote to Table I.

SERIES III. SOIL BACTERIA.

In this series the broth in the flasks was inoculated with the soil, and the bacteria were allowed to grow twelve days. In order to be sure that the new set of sacs were tight, they were inoculated, as in the previous series, with B. typhosus, and grown in sterile broth for forty-eight hours. Those sacs which remained tight were then transferred to the flasks prepared above; so that we have in this series typhoid cultures two days old, and hence

*See footnote to Table I.

thickly seeded, in collodion sacs submerged in flasks containing cultures of mixed soil bacteria grown for twelve days in broth. The samples of soil used are the same as in the previous series. The culture of B. typhosus used here is not the same as that used before, but is the strain described above as B. typhosus, P. D. 1.

The temperature of the experiment was 28°.

TABLE III.

EFFECT OF SOIL ORGANISMS ON B. TYPHOSUS.

No. of Experiment	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing on Agar Plates from One Loop of the Medium							Result
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 Days	3 D.	4 D.	5 D.	6 D.	−Dec. +Inc.
1.......	Soil 1	P.D.1	12 d.	2 d.	28° C.	64,000	..	1,440,000	..	..	..	2,000	−96%
2.......	" 2	" 1	12 "	2 "	28° "	76,000	..	4,000	..	..	..	14	−99%
3.......	" 3	" 1	12 "	2 "	28° "	172,000	..	36,000	..	..	..	4,000	−98%
Control	..	" 1	..	2 "	28° "	440,000	..	∞	..	..	..	∞	+

In this series the soil bacteria had been given abundant opportunity to develop their by-products before B. typhosus was introduced. It is to be borne in mind, however, that there was little or no gain experienced by the extra six days, since it is hardly likely that any considerable amount of growth would have taken place in this last week. It seems probable, then, that the antagonistic action would not necessarily be more marked than in the previous series, even if the other conditions had been identical.

The sacs did not contain any more bacteria per loop in this case than in the preceding case, although the cultures were a day older when brought in contact with their antagonists. It is further to be remembered that the infinity sign has a special and limited meaning.

Thus, while the antagonism exerted by the opposing bacteria is not as marked as in the preceding experiments, it is very apparent and in all cases to be represented numerically as about 95 per cent.

The change in the strain of B. typhosus used may also explain the apparently lessened effect of the antagonism.

SERIES IV. SOIL BACTERIA.

This series was undertaken for the purpose of extending the range of observations on the antagonistic action of soil bacteria to other samples of soils. The source of the material is given here.

Soil No. 5 is from the woods north of Main Hall on the campus, and is the same as No. 3 in Series II and III, except that it was collected late in the fall instead of in the summer.

Soil No. 6 is from a field on the university farm south of Observatory Hill. This soil has been under cultivation several years, and had been plowed in the fall before the sample was taken.

Soil No. 7 is clay excavated in the construction of Agricultural Hall.

Soil No. 8 is a sample of dirt from a macadamized drive running along the university grounds (Linden Drive).

Soil No. 9 is from the back yard of a dwelling-house. (This is "made soil" which for several years had been contaminated with house refuse, the burial of garbage, and horse manure.

Soil No. 10, sand being used for mortar, obtained directly from the pit. Presumably this is like No. 4 in Series III and IV.

The technique in this series was the same as in the preceding experiments.

B. typhosus was inoculated into the sacs, and allowed to grow twenty-four hours before being brought in contact with the opposing germs.

The opposing germs were inoculated at the time the sacs were introduced, and were not given any time for preliminary development.

The culture of the typhoid germ is the same as in the preceding series, viz., the P. D. 1. culture.

The temperature of the experiment is 28°.

The results are shown in Table IV, and graphically in Diagram 2.

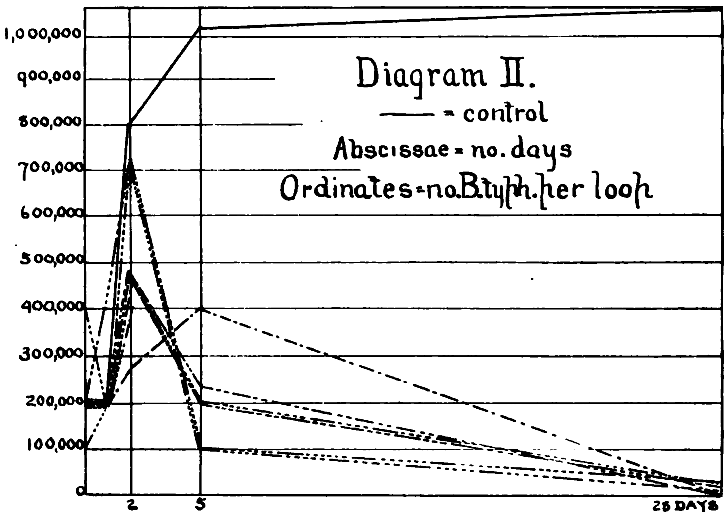
TABLE IV.

EFFECT OF SOIL ORGANISMS ON B. TYPHOSUS.

No. of Experiment	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing in Agar from One Loop of the Medium						Result
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	....	5 Days	28 Days	− Dec. + Inc.
1.......	Soil 5	P.D.1	0	1 day	28° C.	200,000	200,000	28,000	....	400,000	0	− 100%
2.......	" 6	" 1	0	1 "	28° "	200,000	200,000	480,000	....	240,000	0	− 100%
3.......	" 7	" 1	0	1 "	28° "	200,000	200,000	480,000	....	200,000	200	− 99%
4.......	" 8	" 1	0	1 "	28° "	400,000	200,000	480,000	....	200,000	200	− 99%
5.......	" 9	" 1	0	1 "	28° "	200,000	400,000	720,000	....	100,000	−100	− 99%
6.......	" 10	" 1	0	1 "	28° "	100,000	200,000	720,000	....	100,000	200	− 99%
Control	....	" 1	....	1 "	28° "	200,000	200,000	800,000	....	∞	∞	+ 99%

In this series B. typhosus was given every opportunity to maintain its own or even to grow; still it showed a decline which finally amounted to 99 per cent. in all of the samples. The time required to bring this about was considerably greater than in the previous experiments. It is unfortunate that there were no counts

between the fifth and the thirtieth days, so that the exact rate of the decline could have been determined; for it will be noticed that on the fifth day no decrease was observable; indeed in some cases (1 and 2) there appeared to be an actual increase. After the elapse of thirty days the decrease was marked, and in striking contrast to the condition of affairs in the control, where there was no decrease to be detected after a month's time.



SERIES V. SOIL BACTERIA.

The purpose of this series was to retest the soils used in the previous experiment under conditions such that the opposing bacteria would have an opportunity to produce their antagonistic properties before B. typhosus was introduced.

The media in the flasks were inoculated with the various soils and allowed to grow for twelve days. At this time sacs were introduced which had been inoculated with B. typhosus two days before. It thus occurred that B. typhosus, grown for two days in sterile broth, was brought in contact with the opposing bacteria which had grown for twelve days.

The soils were the same as those used in the previous experiment, viz., Nos. 5–10.

The temperature was 28°, as usual.

The culture of B. typhosus used was P. D. 1.

TABLE V.

EFFECT OF SOIL ORGANISMS ON B. TYPHOSUS.

No.	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing on Agar from One Loop of the Medium						Result − Inc. + Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	3 Days			
1.......	Soil 5	P.D.2	12 d.	2 d.	28° C.	480,000	......	40,000	0	......	......	−100%
2.......	" 6	" 2	12 "	2 "	28° "	40,000	......	4,000	3,600	......	......	− 90%
3.......	" 7	" 2	12 "	2 "	28° "	240,000	......	20,000	0	......	......	−100%
4.......	" 8	" 2	12 "	2 "	28° "	480,000	......	240,000	4,000	......	......	− 99%
5.......	" 9	" 2	12 "	2 "	28° "	48,000	......	32,000	∞[1]	......	......	+
6.......	" 10	" 2	12 "	2 "	28° "	co	ntam	inated		......	......	......
Control	......	" 2	......	2 "	28° "	440,000	......	360,000	∞	....	......	+

In a general way the results obtained here are what were to be expected, viz., that when the soil bacteria are given a chance to develop their by-products, and then B. typhosus is brought in contact with them, the result is a marked and rapid decrease in B. typhosus.

In Nos. 1 and 3 (Table V) B. typhosus was entirely killed off in the amount tested. In Nos. 2 and 4 the decrease was marked, but not carried to the extreme as in the above cases.

No. 5 seems to be an exception, and behaves as the controls have behaved heretofore. This is probably accidental, since with the same soil in the previous experiment there is no marked contrast with the other soils, although it is to be noted that this soil is the only one which might be considered a contaminated soil.

SERIES VI. WATER BACTERIA.

In this series the broth is inoculated with water organisms instead of soil organisms.

The water used in one case is that of Lake Mendota, and in the other case a cistern from a private residence.

No opportunity was given the water bacteria to produce their poisons in the broth before the sowing of B. typhosus; indeed, the B. typhosus was inoculated twenty-four hours in advance of the other organisms.

The culture of B. typhosus used was that of P. D. 1.

The temperature was that of 28°.

The results are shown in Table VI:

[1] Probably a contamination.

TABLE VI.

EFFECT OF WATER ORGANISMS ON B. TYPHOSUS.

No.	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing in Agar from One Loop of the Medium						Result + Inc. − Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	5 Days	6 Days	30 Days	
1.......	(Water) Lake	P.D.1	0 d.	1 d.	28° C.	200,000	200,000	400,000	400,000	......	0	−100%
2.......	Cistern	" 1	0 "	1 "	28° "	200,000	200,000	800,000	400,000	......	0	−100%
3.......	Lake	" 1	12 "	2 "	28° "	300,000	......	640,000	......	64,000	......	− 78%
4.......	"	" 1	12 "	2 "	28° "	120,000	......	64,000	......	64,000	......	− 47%
5.......	"	M. H.	0	0	28° "	5,200	9,600	3,300	200	100	0	−100%
Control	....	P.D.1	......	......	28° "	200,000	200,000	∞	∞	∞	∞	+

It would appear here, as in the previous experiments on soil bacteria, that B. typhosus is at a distinct disadvantage when grown under conditions which permit the action on it of the by-products of certain other bacteria found in nature.

That this antagonism is not immediately demonstrable is undoubtedly due to the fact that B. typhosus was given an opportunity to develop before the other bacteria were sown. It is not surprising, then, that there was an increase for the first few days. At the end of the month, however, there was a very marked antagonism, the decrease amounting to practically 100 per cent. in the first two and last cases, and 78 and 47 per cent. in the other two cases respectively.

In the third and fourth cases it is to be noticed that the conditions are different from those in the first two cases. Here the opposing bacteria were given a distinct start, and the number of determinations were fewer and extend only over a week's time, and not a month as in the preceding cases.

SERIES VII. WATER BACTERIA.

There being an antagonism distinctly observable when broth is seeded with the bacteria from water, it was decided to determine whether the antagonistic substances are developed when grown in water instead of broth. Flasks were, therefore, fitted up with collodion sacs and filled with lake water and sterilized in the autoclave, and the sacs were seeded with B. typhosus, and the water in one of the flasks was inoculated with water bacteria by adding a few drops of raw lake water. The other flask was not inoculated.

The culture used was the M. H.

The temperature was that of the room in summer, 20–22°.

The results are indicated in the following Table VII:

TABLE VII.

Effect of Sterile and Raw Water on the B. typhosus.

No.	Source of Organisms		Period of Prelim. Cultivation		Temperature	No. of Colonies of B. typhosus Developing on Agar from One Loop of the Medium						Result +Inc. −Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 D.	4 D.	6 D.	30 D.	
1.....	Sterile water	M. H.	0	0	20–22° C	4,400	1,600	940	590	270	0	−100%
2.....	Raw water	M. H.	0	0	20–22° C	2,900	590	380	30,000	30,00	Very few	−100%

There is in sterile water a gradual, but distinct, decrease, which in a relatively short time amounts to extinction.

In the raw water the decline was checked at the end of the second day, and the number began to increase and was maintained for some days, and then began to decline again; but at the end of one month's time B. typhosus had not entirely disappeared. During this time, however, the number of water bacteria in the flask outside of the sac had increased to an enormous extent, as they always do under similar circumstances, *i. e.*, when water is inclosed in a small space and kept at a temperature which will permit of the growth of bacteria. It is true, then, that the water in the flask after the lapse of a few days is a very different water from what it was when B. typhosus was first introduced.

SERIES VIII. WATER BACTERIA.

In view of the results obtained in the previous series, it was decided to try to grow B. typhosus in a collodion sac in water kept under conditions which would keep down its germ content; in other words, to grow B. typhosus in the lake. For various reasons the sacs were not put in the lake itself, but were kept in the laboratory sink, under conditions, to be presently explained, which were, so far as can be seen at the present time, exactly similar in effect to those which obtained in the lake.

This procedure was intended to keep the water in motion, and thus prevent the enormous multiplication of the germs which always occurs when water taken from a large body of water is confined in a small vessel. For this purpose an apparatus was arranged as indicated in Fig. 6.

The collodion sacs were made as usual and sterilized in flasks of water. The sacs with their sterile water content were then placed in the running-water, where they were left without inoculation for twenty-four hours, Cultures of the water in the sacs were then made in order to determine whether the sacs were tight or not. In some cases it was found that they were not

tight, and in these cases they were replaced with new ones, which were in turn tested in the same way. When the sacs were found to have remained in running water for twenty-four hours, without allowing the water bacteria to gain entrance, they were inoculated with B. typhosus. Cultures were made at this time and at regular periods for twenty days.

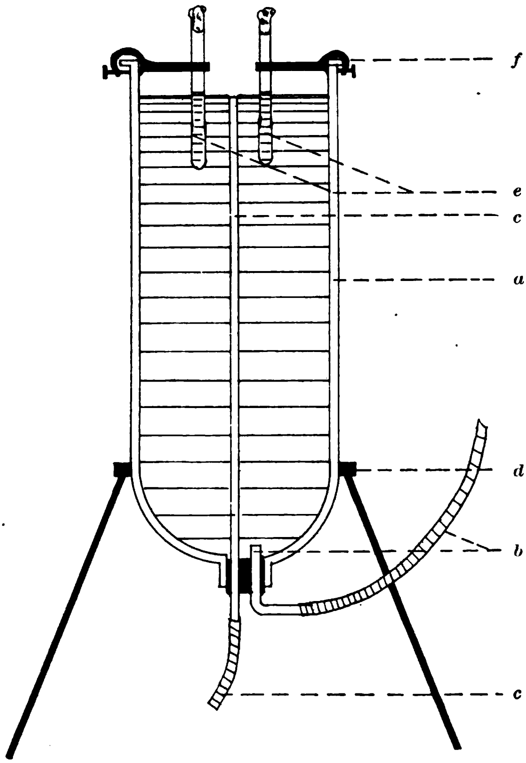


FIG. 6.—Apparatus for immersing collodion sacs in running water. *a*, glass bell jar; *b*, inlet tube; *c*, outlet tube; *d*, tripod; *e*, collodion sacs sealed on test-tubes and inoculated with the typhoid germ, held in place by the iron clamps *f*.

The experiment was conducted in duplicate, as will be seen, and was also accompanied by a control in sterile water, which was also conducted in duplicate. The culture was the M. H.

The temperature of the water, of course, varied. A thermometer was kept in the apparatus all of the time, and the temperature was read each morning and recorded.

The results are shown in Table VIII.

In the case of the controls, or boiled water, there was a decline in the first day or two, which was followed by a marked and very sudden increase, and then a gradual decline, which at the end of twelve days amounted to extinction, so far as the amount tested was concerned.

In the raw, or running, water there was apparently no decline, but in one of the two cases there was an increase observed in the first few days, and then a decline which reached a low point in seven days, or five days sooner than in the case of boiled water. It must be noted, however, that B. typhosus persisted quite as long in this case as in the boiled water; indeed, these figures seem to indicate that at the end of twenty days they were more numerous, per loop of material, than were the typhoid germs at the same time in the boiled water.

TABLE VIII.

EFFECT OF STERILE AND RAW LAKE WATER ON THE B. TYPHOSUS.

No. of Experiment	Opposing Medium	Culture of B. typhosus used	Temperature	Number of Colonies of B. typhosus Developing on Agar from one Loop of Medium									Result +In. −De.
				0	1 D.	2 D.	5 D.	7 D.	11 D.	12 D.	16 D.	20 D.	
1	Boiled water	M. H.	18–23	600	360	4,750	400	100	6	2	0	0	−100%
2	Boiled water	M. H.	18–23	450	125	325	6,500	400	600	15	1	1	−100%
3	Raw water	M. H.	18–23	300	300	480	200	10	...	...	...	5	− 98%
4	Raw water	M. H.	18–23	200	175	135	85	16	16	10	10	10	− 95%

No very definite conclusions seem warranted from these results. It appears, however, that there were conditions in the boiled water which permitted a rapid and extensive development for a few days. There seems to have been no shortening of the life of B. typhosus in raw, or running water. If the water bacteria exert a prejudicial effect on B. typhosus, either it is so slight that it cannot be detected by this means, or it does not pass through the collodion sac. This result is not in accord with numerous investigators on this subject.

GENERAL DISCUSSION OF RESULTS.

The results of the experiments already recorded show that there is a marked antagonism exerted by soil and water bacteria on B. typhosus when the same are grown in broth and B. typhosus are immersed therein, for, whereas B. typhosus in the collodion sac in sterile broth grows rapidly and maintains itself in enormous numbers for long periods of time, it rarely multiplies to any extent in the broth saturated with the by-products of these bacteria, and invariably shows, usually in a few days, a rapid decline, which in the course of a few days, or weeks at most, amounts to practical extinction in the quantities tested.

The antagonistic effects are produced more rapidly, and the effect is more marked, when the opposing organisms are allowed to develop their by-products before B. typhosus is introduced; and, *vice versa*, where B. typhosus is allowed to develop before

the opposing germs are introduced, some considerable time is required to demonstrate the antagonism, and the action is slower and less marked. The time required to develop the antagonistic by-products is comparatively short, very positive results being obtained in forty-eight to seventy-two hours. The influence which the prolongation of the period of incubation of the opposing bacteria has on the rapidity of the extinction of B. typhosus is well shown in the following table. The B. typhosus was grown in the collodion sac, as in the preceding experiments, and the opposing organisms were from soil No. 3. The temperature was 28°, and the culture of B. typhosus was the M. H.

TABLE IX.

THE EFFECT OF PROLONGING THE PERIOD OF INCUBATION OF THE OPPOSING BACTERIA ON RATE OF EXTINCTION OF B. TYPHOSUS.

No. of Experiment	Days of Preliminary Cultivation		Day before Extinction	Per Cent. Decrease
	B. typhosus	Opposing Bacteria		
1	0	12	2	100
2	0	6	5	100
3	2	0	7	100

A question of considerable importance is this: Do soil and water bacteria growing in their natural habitats produce by-products in sufficient quantities to saturate these substances, and thus give to them a germicidal property? This has been very frequently tested for the water bacteria, and while there seems to be no consensus of opinion as to the germicidal properties of the natural waters, there is abundant proof that raw waters are not suited for the development of B. typhosus, but the heating of the same in the process of sterilization renders the water capable of sustaining the organism for a longer time, and under some conditions permits the multiplication of the typhoid germ in them. This point does not seem to have been determined for soils. The following experiment was made in this connection: Soil was collected from a plowed field. The bacteria in this soil had already been shown to possess antagonistic properties, since it

was the same as soil No. 6 in Series V. One kilogram of this soil was placed in a large funnel, and to it one liter of boiled tap water was added. The first 300 c.c. were caught and filtered through a Pasteur-Chamberland filter (No. F) and distributed into two small, sterile flasks. One of these was afterward run through an autoclavê. A similar flask of autoclaved tap water was used as a control. These flasks were heavily seeded with B. typhosus (P. D. 1.) and put at a temperature of 38°, so that if there were no antagonistic substances present, B. typhosus would probably multiply. The results obtained follow:

TABLE X.

CAREER OF B. TYPHOSUS EXPOSED TO RAW, FILTERED SOIL LEACHINGS; BOILED, FILTERED LEACHINGS; AND BOILED TAP WATER.

No. of Experiment	Description	No. Typhoid Colonies from Two Loops		
		0	2 Days	3 Days
1	Filtered leachings............	500	12,000	35,000
2	Filtered leachings (autoclaved)	500	20,000	20,000
3	Tap water (autoclaved).......	2,000	70	17

From the data recorded in the above table there seems to be no evidence whatever that there are any substances present in the soil which are antagonistic for the typhoid germ. There is apparently no difference between the raw and autoclaved leachings, and at 38° there is a marked increase in the number of germs in three days. This is in strong contrast with the condition of affairs in the case of the autoclaved tap water, where under identical conditions there is a marked decrease. This difference is probably to be explained by the presence of a large amount of organic matter in the soil leachings which furnishes conditions favorable for the rapid development of B. typhosus. It seems probable, then, that the antagonism which a soil may possess for B. typhosus is due to the bacteria present and the by-products which they may produce in the immediate presence of the typhoid germ, and not to by-products previously formed.

The bacteria which are antagonistic to B. typhosus seem to

be widely distributed in the soil and water. So far, they have been found in practically all of the samples examined. These include soils from various sources, as woods, grass plots, plowed ground, road dust, clay from several feet below the surface, and sand, as well as various waters.

ANTAGONISM EXHIBITED BY PURE CULTURES OF CERTAIN BACTERIA.

In the previous section the fact is shown that when B. typhosus is grown on one side of a permeable membrane, as a collodion film, and a mixed culture of soil or water bacteria on the other side, B. typhosus is not only unable to grow, but in most cases rapidly dies out. The question then naturally arose whether it was possible to secure single species of bacteria which, when grown in pure culture, would produce a similar result. With this end in view the following experiment was performed:

After Series II (p. 610) had been running for eight days, a number of agar plates were made from each flask in order to isolate the various bacteria present for further study. But as there was an interruption of the work at this time, the plates remained in the ice-chest for about two months. Subcultures were then made from the various colonies, and after they had grown for a few days on agar slopes they were inoculated into flasks fitted with collodion sacs. The sacs had been inoculated with B. typhosus twenty-four hours earlier. The stock of B. typhosus was P. D. 1. The temperature of the experiment was 28°. Cultures from seven different colonies were tested in this way. In regard to the results it may be stated, without going into greater detail, that there was no decline in the numbers, as was expected, but that there was in all cases a four, or more, fold increase and that these numbers were maintained for at least one month. These results may be accounted for on any of the following suppositions: First, it is possible that the particular organism possessing antagonistic properties was not obtained in the plate cultures. But it is to be remembered that the plates were made when the antagonistic properties were very strong, and it would be expected that this organism would be more or less

abundant in the mixture. Second, it is possible that the organisms, although normally antagonistic, may have lost their peculiar properties through the long standing in the plate culture. Third, it may also be true that the antagonism exerted in the former experiments is due, not to a single organism, but the combined action of two or more different species.

While the last experiment was in progress, and as a check on the same, it was determined to test the effect of growing pure cultures of certain bacteria in close proximity to B. typhosus in solid media, somewhat after the method used by Garré. (Method 1, p. 602.) Therefore a series of agar plates were made which were heavily seeded with B. typhosus. After the agar had thoroughly solidified, streaks were made on the surface of the agar with the organisms to be tested. These were new cultures from soil No. 4, the same as that used in Series II, a sample of which had been kept. After twenty-four hours at 28° it was found that the bacteria growing in the streaks on the surface of the media had in one case seriously interfered with the growth of B. typhosus in the agar immediately beneath. In this case case B. typhosus had not developed into visible colonies beneath the streak and for some distance on each side of the streak.

By this means an organism was discovered which showed strong antagonistic properties. This culture proved to be, on further study, the "potato bacillus," or B. vulgatus Trevisan.

A number of pure cultures were now tried in the same way. These included the following well-known bacteria: Pseudomonas fluorescens (Fluegge) Migula (B. fluor. liquefaciens); Pseudomonas aeruginosa (Schroeter) Migula (B. pyocyaneus); Pseudomonas putida (Fluegge) Migula (B. fluorescens putidus); Bacillus prodigiosus (Ehrenberg) Fluegge; Bacillus vulgaris (Hauser) Migula (Proteus vulgaris).

In this way an antagonism was detected for B. typhosus in the case of three out of five germs, viz.: Ps. fluorescens, Ps. putida, and B. vulgaris. A number of experiments with these three germs, together with B. vulgatus, were carried out, and the antagonism which they exert on B. typhosus was very definitely shown.

Two courses were now open: the various bacteria in the soils already examined could be isolated, and the relation of each individual species to B. typhosus could be determined and their distribution studied; or the organisms already found could be studied for the purpose of determining the extent of the antagonism, the influence of various factors in modifying the antagonism, and finally the cause of the antagonism could be investigated. The latter course was determined on, and it is now proposed to discusss the results in detail.

ANTAGONISM EXHIBITED BY B. VULGATUS TREVISAN.

This organism is usually designated, incorrectly, by the trinomial, Bacillus mesentericus vulgatus Fluegge. The common name is the "potato bacillus."

The culture used was isolated from soil No. 4 in Series II (p. 611), and at first designated by number as 59-4, but upon further study it has proved to be B. vulgatus.

No reference in literature has been found to the antagonistic action of this germ for B. typhosus, but, as the following experiments show, it possesse a strong antagonistic action.

Agar plates were heavily seeded with B. typhosus, and when the agar was hard, it was streaked with B. vulgatus. The results follow:

TABLE XI.

ANTAGONISM EXERTED BY B. VULGATUS.

No. of Exp'm't	Temperature	Kind of Medium	Results
1	28	Nutrient agar	Antagonism apparent.
2	28	"	Marked antagonism.
3	28	"	Strongly marked (¼ inch clear agar around streak).
4	28	Hiss's medium	Strongly marked antagonism.

The presence of an antagonism was also shown by means of the double-plate method (No. 6, p. 604) where B. typhosus repeatedly failed to grow on the side of the plate seeded with B. vulgatus (see Plate XVIII, Fig. 1, *A*).

Again, the agar-block method was used. The B. typhosus was inoculated into agar blocks, and these were then put into broth cultures of the potato bacillus. Repeated trials have shown that under these conditions the typhoid germ invariably fails to grow. (See Plate XVIII, Fig. 2, *D*.)

This series of experiments shows clearly the antagonism which is exerted, since in all cases controls were made in which B. typhosus was grown on itself.

TABLE XII.

INFLUENCE OF PRELIMINARY CULTIVATION OF B. VULGATUS ON DEVELOPMENT OF ANTAGONISM.

No. of Experiment	Temperature	Time Allowed B. vulgatus to Grow before B. typhosus Is Seeded	Results
1	28	0 hours	No antagonism.
2	28	0 "	Slight (if any).
3	28	6 "	Slight antagonism.
4	28	48 "	Marked antagonism.

The above table brings out another fact, and that is that it requires some time for B. vulgatus to develop its antagonistic products. Thus it happens that, if B. typhosus is seeded at the time that the other germ is seeded, it has time to develop into a visible streak before its growth is checked. This happened in Nos. 1 and 2. When, however, B. vulgatus is given a start, it invariably prevents the development of B. typhosus.

The antagonism is also demonstrable when this germ is grown in broth and B. typhosus is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28°, and the culture of B. typhosus was the P. D. 2.

TABLE XIII.

ANTAGONISM OF B. VULGATUS.
(Collodion Sac Method.)

PERIOD OF PRELIMINARY CULTIVATION		NO. OF COLONIES OF B. TYPHOSUS PER LOOP						
B. vulgatus	B. typhosus	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	1,400	....	360	....	....	....	40

ANTAGONISM EXHIBITED BY BACILLUS VULGARIS (HAUSER) MIGULA.

This organism is usually known as Proteus vulgaris;* occasionally as Bacillus proteus.†

There seems to have been no experimental work on the antagonism exerted by B. vulgaris for B. typhosus recorded, although the idea is quite general that the group of organisms to which this belongs, *i. e.*, the Proteus group, does possess antagonistic properties for the typhoid germ. For instance, Sidney Martin,[5] in giving the reasons for the disappearance of the typhoid

* HAUSER, *Ueber Fäulnissbakterien*, 1885.

† TREVISAN, *Genera*, 1889.

germ from unsterilized, virgin soil, remarks that the disappearance seems to be due to the antagonism of the soil micro-organisms, and stated that "there is some evidence to show that, under the conditions of the experiment, the disappearance of the bacillus [B. typhosus] has taken place *pari passu* with the increase of the number of the putrefactive bacteria."

The source of the particular culture used was the Laboratory of the Johns Hopkins Hospital, where it was known as 22*b*. This germ was isolated from several of the soils used.

Experimental work.—Agar plates were heavily seeded (Method 1, p. 602) with B. typhosus, and when the medium had hardened the surface was streaked with B. vulgaris. The plates were incubated at 38°. Twenty-four hours later it was invariably found that there was a marked antagonism for B. typhosus. Usually the colonies were small under the streak and for some distance each side of the streak. (See Fig. 2.) In some cases the typhoid colonies under the streak of B. vulgaris did not develop at all, and the agar remained clear for a short distance each side of the streak. The results obtained in this series of experiments is shown in the following table:

TABLE XIV.

ANTAGONISM OF B. VULGARIS.

No. of Experiment	Temperature	Kind of Medium	Results
1	38	Nutrient agar	Marked antagonism
2	38	" "	" "
3	36	" "	" "
4	38	" "	" "
5	38	" "	" "

The antagonism was also tested by the double-plate method, and the results obtained are shown in the following table (see also Fig. 5, *B*):

TABLE XV.

ANTAGONISM OF B. VULGARIS TESTED BY THE DOUBLE-PLATE METHOD.

No. of Experiment	Temperature	Medium	Time Allowed B. vulgaris before seeding B. typhosus	Results
1	28	Nutrient agar	0 hours	Marked
2	28	" "	0 "	"
3	28	" "	6 "	"
4	28	" "	6 "	"
5	28	" "	48 "	Strong

It will be noticed here, in contrast to the condition of affairs under B. vulgatus, that B. vulgaris develops its antagonistic properties immediately and, in the experiments performed here, there was no case where B. typhosus was able to grow on the seeded half of the plate.

This organism has been grown in broth, and in these cultures agar blocks have been immersed, and it has been shown repeatedly that B. typhosus will not develop in the by-products of this germ. (See Plate XVIII, Fig. 2, *E*.)

The antagonism is also demonstrable when this germ is grown in broth and B. typhosus is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28° and the culture of B. typhosus was the P. D. 2.

TABLE XVI.

ANTAGONISM OF B. VULGARIS.
(Collodion Sac Method.)

Period of Preliminary Cultivation		No. of Colonies of B typhosus per Loop						
B. vulgaris	B. typhosus	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	3,200	....	3,600	....	....	....	1,500

ANTAGONISM EXHIBITED BY THE PSEUDOMONAS FLUORESCENS (FLUEGGE) MIG.

This germ is usually spoken of as the Bacillus fluorescens liquefaciens.* It has also been described as B. viscosus† and B. Fluorescens nivalis.‡

The particular cultures used here were one isolated from the water in Lake Mendota and one obtained from Professor Jordan, of the University of Chicago.

The antagonism exhibited by Ps. fluorescens for B. typhosus was apparently first described by Olitzky.[3] This investigator found that the organism in question exerted a strong restraining influence on B. typhosus, and suggested that this fact might have practical hygienic importance. The methods used by this worker were slight modifications of Garré's methods.

Laws and Andrews,[4] working on the duration of the life of B. typhosus in sewage, tested the effect of the presence of different organisms on B. typhosus, and among them Ps. fluorescens, and concluded that it had no effect on B. typhosus.

* FLUEGGE, *Mikroorganismen*, 1886, p. 289.
† FRANKLAND, *Ztschr. f. Hyg.*, 1887, 6, p. 39.
‡ SCHMELK, *Centralbl. f. Bakt.*, 1888, 4, p. 544.

Horrocks,[7] working on similar lines, found that he could not isolate B. typhosus from sewage after seven days, when Ps. fluorescens was present, but could isolate it from sterile sewage two months after inoculation. He did not, however, feel sure but that some typhoid colonies had escaped him in the first case. He also found that B. typhosus would not grow on the surface of gelatin which had already yielded a growth of Ps. fluorescens.

The effect of this organism on B. typhosus has been reinvestigated by means of the new methods already described.

Agar plates were seeded with B. typhosus, and when the agar was well hardened Ps. fluorescens was streaked over the surface. In most cases it was found upon development that the colonies under and immediately around the streak of Ps. fluorescens had either not grown or were much smaller than in other parts of the plate. The following table gives the data in detail:

TABLE XVII.

ANTAGONISM EXERTED BY THE PSEUDOMONAS FLUORESCENS DEVELOPING ON AN AGAR TYPHOID PLATE.

No. of Experiment	Temperature	Medium	Result
1	28°	Nutrient agar	Antagonism apparent
2	28	" "	" "
3	28	Sugar-free agar	" "
4	28	" "	" "

From these experiments it seems fair to conclude that when the two organisms are grown as in the preceding experiments there is a marked inhibiting effect exerted by Ps. fluorescens on B. typhosus which frequently extends through several millimeters of agar.

The difficulty which B. typhosus has in growing in the presence of Ps. fluorescens is further shown in the following experiments with the double-plate method (see Plate XVIII, Fig. 1, *C*):

TABLE XVIII.

ANTAGONISM OF PS. FLUORESCENS TESTED BY DOUBLE-PLATE METHOD.

No. of Experiment	Temperature	Time Allowed Antibiont to Develop before Seeding B. typhosus	Result
1	28°	0 hours	Very slight antagonism
2	28	0 "	Slight antagonism
3	28	6 "	Marked antagonism
4	28	48 "	Very marked antag.

In addition to the fact that there is an antagonism, the further fact is brought out that time is required for the development of the maximum amount of antagonism.

The antagonism has been further tested by means of the agar-block method, and in no case was B. typhosus able to grow in the by-products of this germ (see Plate I, Fig. 2, *F*).

The antagonism is also demonstrable when this germ is grown in broth and B. typhosus is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28° and the culture of B. typhosus was the P. D. 2.

TABLE XIX.

ANTAGONISM OF PS. FLUORESCENS.
(Collodion Sac Method.)

PERIOD OF PRELIM. CULTIVAT'N		NUMBER OF COLONIES OF B. TYPHOSUS PER LOOP						
Ps. fluorescens	B. typhosus	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
30 days	0	720	0	...	...	0	...	...

ANTAGONISM EXHIBITED BY PSEUDOMONAS PUTIDA (FLUEGGE) MIGULA.

This organism is usually known as the Bacillus fluorescens putidus Fluegge, and differs slightly, if at all, from the Bacterium immobile (B. fluorescens non-liquefaciens) except in motility.

The culture used here was one obtained from Professor Jordan, of the University of Chicago.

Garré[2] has called attention to an antagonism exerted by Ps. putida on the B. typhosus, but apparently no one else has studied it.

The antagonism has been studied by means of the double-plate method and found to be marked, although apparently not as strong as in the case of Pseudomonas fluorescens (see Plate I, Fig. 1, *D*).

In the agar blocks there is usually no growth along the streak, although occasionally a slight growth does occur as in the tube represented in Plate I, Fig. 1, *G*.

An antagonism is demonstrable by means of the collodion-sac method, as the following table will show. The temperature of this experiment was 28°, and the culture of B. typhosus used was P. D. 2.

TABLE XX.

ANTAGONISM OF PS. PUTIDA.
(Collodion Sac Method.)

PERIOD OF PRELIM. CULTIVAT'N		NUMBER OF COLONIES B. TYPHOSUS PER LOOP						
Ps. putida	B. typhosus	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	1,200	...	650	...	...	...	150

GENERAL DISCUSSION OF RESULTS.

These organisms all show a marked antagonism for B. typhosus when tested as above described. Of the four Pseudomonas fluorescens exhibits the strongest antagonistic properties, although it requires some time to develop this properly. B. vulgaris acts more promptly than the others, but the antagonistic substance does not diffuse to so great a distance in the medium.

These germs are among the most common and widely distributed of the soil and water bacteria.

These germs all produce substances which pass through a collodion film. The results obtained here by this method, however, have not been as striking in case of B. vulgatus and B. vulgaris as in the case of the mixed cultures; partly, no doubt, because the cultures had been grown for a longer time on artificial culture media; and, furthermore, it is quite likely that the greater antagonism in the mixed cultures may have been due to the combined action of two or more species.

EFFECT OF VARIOUS AGENTS IN INFLUENCING ANTAGONISM.

A series of experiments were undertaken to determine the effect of various agents in influencing the antagonism exerted by these germs.

INFLUENCE OF INCUBATING TEMPERATURE ON THE DEVELOPMENT OF ANTAGONISM.

Double plates were made and one-half seeded with these organisms, and then a few hours later both sides were streaked with B. typhosus. The plates were kept at the temperature of the ice-chest (10–12°), room temperature (17–20°), low incubator (28°), and blood heat (38°), with the following results:

TABLE XXI.

EFFECT OF INCUBATION TEMPERATURE ON ANTAGONISM.

ORGANISM	TEMPERATURE			
	10–12°	17–22°	28°	38°
B. vulgatus	0	0	0	0
B. vulgaris	0	0	0	0
Ps. fluorescens	0	0	0	0
Ps. putida	0	0	0	0

0 means no growth.

The effect of incubation temperature was tested in another way, as follows:

Tubes of neutral broth were inoculated with the four organisms and grown at the different temperatures. These were allowed to grow six days, and at the end of that time there was a good growth even in those tubes kept at the ice-chest temperature. All of these tubes were then heated to 60° for ten minutes.

An agar block seeded with B. typhosus was then placed in each tube, and all placed at the temperature of 38°.

The purpose of the heating was to kill the vegetative forms and thus prevent, as much as possible, the formation of the antagonistic substance at 38°. The heating had been found not to interfere with the action of the antagonistic substance already formed. Twenty-four hours later the following results were obtained:

TABLE XXII.

EFFECT OF INCUBATION TEMPERATURE ON ANTAGONISM.

ORGANISM	TEMPERATURE			
	10–12°	17–22°	28°	38°
B. vulgatus	+	+	0	0
B. vulgaris	0	0	0	0
Ps. fluorescens	+	+	0	0
Ps. putida	++	+	Contaminated; had a pellicle	+

+ means that the streak was visible.
++ means that the streak was distinctly visible.
0 means that the streak was not visible.

From these results it would appear that the temperature variations do not have any appreciable effect, as measured by the means at command; that the temperatures which permit growth also permit the production of the antagonistic substances.

ACTIVITY OF ANTAGONISTIC SUBSTANCES AT DIFFERENT TEMPERATURES.

The germs here were allowed to grow under as nearly as possible optimum conditions, and when they had had the opportunity to develop their by-products, the broth cultures were divided and a portion of each put at the different temperatures. At this time an agar block, seeded with the typhoid germ, was placed in each, and they were allowed to remain six days at the different temperatures. The results were as follows:

TABLE XXIII.

ACTIVITY OF ANTAGONISTIC SUBSTANCES AT DIFFERENT TEMPERATURES.

ORGANISM	TEMPERATURES			
	10–12°	17–22°	28°	38°
B. vulgatus	++	++	+	0
B. vulgaris	++	++	0	0
Ps. fluorescens	++	++	0	0
Ps. putida	++	++	+	0

++ means slight growth.
\+ means that the streak was visible.
0 means that the streak was not visible.

This indicates that the action of these antagonistic substances varies somewhat with the temperatures, that at high temperatures —that of the body—the action is most pronounced, and that at the temperature of the ice-chest it is so delayed that B. typhosus has an opportunity to develop. This appears to be a point of great interest, and offers at least a partial explanation of the singular fact that water supplies which become contaminated in cold weather retain their power of producing infection for a much longer time than when the contamination occurs in warm weather.

HEAT STABILITY OF ANTAGONISTIC SUBSTANCES.

The attempt to determine the temperature which was destructive to these substances was made as follows:

A large flask of broth was seeded with the Ps. fluorescens and kept at 28° for about two weeks. From this flask a number of sterile test-tubes were filled and heated at various temperatures—50°, 55°, 60°, 65°, 70°, 75°, 80°, and 100°—for ten minutes. These tubes then had placed in each an agar block seeded with B. typhosus. They then were incubated at 38°. The typhoid germ failed to develop in any, although it grew well in a tube of sterile broth used as a control.

Agar tubes were then melted, cooled, seeded with Ps. fluorescens, and allowed to grow as shake cultures for a week. They were then heated in the autoclave at 120° for ten minutes. The melted culture was then allowed to solidify in a sloping position, and later streaks of B. typhosus were made and the cultures incubated at 38°. The typhoid streak did not develop, showing that the restraining substance had not been destroyed.

The above experiments show that these antagonistic substances are thermo-stable, being able to withstand the action of 120° C. for ten minutes at least; and in this connection it is interesting to note that Abbott and Gildersleeve[16] have recently shown that the by-products of some of the common bacteria may be heated to 100° C. for fifteen to thirty minutes without changing their hemolytic activities.

INFLUENCE OF DEXTROSE IN THE MEDIUM.

Double plates were made, and the organism whose antagonistic properties was to be tested was seeded on one half in ordinary nutrient media, made from extract of beef, and therefore, practically sugar free. In some cases sugar-free agar was used, made according to Smith's well-known directions. On the other half the organism was sown in agar containing 1 per cent. of dextrose. A few hours later the plates were streaked with B. typhosus and incubated at 28°.

No very definite results could be obtained with any of the above organisms, although an organism that did not antagonize the typhoid germ when grown on sugar-free media was strongly antagonistic on the dextrose media.

A further attempt was made to study this with agar blocks, but it was likewise impossible to determine any influence exerted by the presence of dextrose.

INFLUENCE OF OXYGEN ON DEVELOPMENT OF ANTAGONISTIC SUBSTANCES.

Double plates were made, and one set kept as usual in the air, and another in a Novy jar from which the oxygen was absorbed by the use of an alkaline pyrogallic acid solution. Growth in the absence of oxygen did not seem to affect in any way the development of the antagonistic substances, which were apparently as strong under one condition as the other.

INFLUENCE OF REACTION OF MEDIUM ON THE DEVELOPMENT OF ANTAGONISM.

To test this the different bacteria were grown in broth to which varying amounts of normal acid and alkali had been added. The amount is indicated in the Fuller scale. After the cultures had been incubated several days, agar blocks seeded with B. typhosus were added, and the following results obtained:

TABLE XXIV.

INFLUENCE OF REACTION OF MEDIUM ON ANTAGONISM.

Reaction of Medium Expressed in Per Cent. Normal Solutions	+1.5	0	−1.5	−3	−5
B. vulgatus	−	0	0	−	−
B. vulgaris	−	0	0	−	−
Ps. fluorescens	−	0	0	0	0
Ps. putida	−	+	+	+	−

− means no growth of bacterium and therefore agar block not added.
0 means streak was not visible.
\+ means that streak was visible, but no growth.

These results would indicate that the reaction of the culture medium was not effective in influencing the production or action of the antagonistic substances.

GENERAL DISCUSSION OF RESULTS.

In reviewing the behavior of these organisms under the conditions studied above, it would seem that the various agents have little, if anything, to do in influencing the development of the antagonism which they exhibit for B. typhosus.

Conditions which favor the growth of the various bacteria undoubtedly favor the development of the antagonistic properties,

in that they hasten their production, but there is no evidence at hand to show that these substances are not produced whenever a good growth occurs.

While, then, the antagonistic properties are produced in all well-developed cultures, they do not seem to be equally active under all conditions, and especial attention is called to the fact, already developed, that at low temperatures the antagonism exhibited is appreciably less than it is at higher temperatures—a fact apparently of great hygienic significance.

POSSIBLE EXPLANATIONS OF ANTAGONISM.

THEORY OF EXHAUSTION OF FOOD SUPPLY.

One of the first theories advanced to explain the antagonism exerted by one bacterium for another was that it was due to an exhaustion of the food supply. Olitzky[3] controverted this and presented the evidence that Micrococcus aureus would grow on media which had supported a previous crop of bacteria, but which would not permit the growth of B. typhosus. The following experiment also clearly demonstrates that the food-exhaustion theory is not a sufficient explanation. Ps. fluorescens was grown in broth for some days, and then the culture was pasteurized, and part of it was placed in a sterile test-tube, and part in an equal quantity of sterile broth. To each test-tube was then added an agar block of B. typhosus. B. typhosus failed to grow in either, although it grew well in a control tube of sterile broth, thus showing that growth is prevented in the presence of abundant food material.

THEORY OF ENZYME ACTION.

The theory that the antagonistic action is due to an enzyme is one that is frequently held. This, of course, could not be the explanation in all cases, because organisms, as Ps. putida, which do not produce enzymes, exert an antagonistic action. In the case of liquefiers, however, it might be explained in this way.

Houston,[17] in discussing the survival of pathogenic organisms after passing through bacterial filter beds, says:

> . . . . thirdly, that the balance of evidence points to the probability that some, at all events, of the pathogenic organisms are crowded out in the

struggle for existence in a nutritive medium containing a mixed bacterial flora, their vitality being weakened or destroyed by the enzymes of Saprophytic Species.

It seems very doubtful if this antagonism is due to enzymes.

The commercial enzymes, in powdered form were tested on B. typhosus by means of the double-plate method, and in no case was there a definite antagonistic action observed. The enzymes tested were Taka diastase, pepsin, trypsin, and pancreatin.

Nor would it be possible to explain the antagonistic action observed in the case of the collodion sacs on this theory, since the enzymes are colloidal in nature, and they do not pass through the collodion film, as the following experiment shows:

A collodion sac was prepared in the usual way, and sterilized in a flask of gelatin instead of broth, so that when the gelatin had hardened the sac was imbedded in it. The sac was then inoculated with a liquefying organism (Pseudomonas aeruginosa) and allowed to stand for some time. This finally amounted to about six months. During this time there was abundant growth in the sac, but no effect on the gelatin outside. To show that the culture had not lost its power to produce an enzyme, or had not become otherwise modified, some of the culture was introduced into an ordinary gelatin tube, where it produced the ordinary, characteristic changes, and then the gelatin outside of the sac was inoculated by rupturing the sac, when there took place the rapid liquefaction of the gelatin in the flask. It, therefore, seems quite certain that the enzymes cannot pass through the collodion sac, and so are not capable of explaining the action.

It is further shown by the followimg experiment that the antagonistic action in the case of Ps. fluorescens, at least, cannot be due to the enzymes produced.

It will be recalled that this substance is thermo-stable, but it can be readily shown that the proteolytic action of the products of this germ, as tested by means of milk-agar blocks, is largely, if not completely,* destroyed by an exposure to 75° C. for ten minutes. This seems sufficient proof that the substance is not identical with the proteolytic enzyme produced by this organism.

* In this connection it is worthy of note that these results do not agree with those of Abbott and Gildersleeve.[16]

THEORY OF SPECIFIC POISONS.

Newman,[18] in his book on bacteria, says:

In several of the most recent of the admirable reports of Sir Richard Thorne, issued from the medical department of the Local Government Board, we have the record of a series of experiments performed by Dr. Klein into this question of the antagonism of microbes. From this work it is clearly demonstrated that whatever opposition one species affords to another it is able to exercise by means of its poisonous properties. These are of two kinds. There is, as is now widely known, the poisonous product named the toxin. There is also in many species, as Dr. Klein has pointed out, a poisonous constituent or constituents included in the body protoplasm of the bacillus, and which he therefore terms the *intracellular poison.* Now, whilst the former is different in every species, the latter may be a property common to several species. Hence those having a similar intracellular poison are antagonistic to each other, each member of such a group being unable to live in an environment of its own intracellular poison.*

While this explanation is an attractive one, it does not seem to satisfy the conditions. In the first place, if the poisonous substances were intracellular, one would expect them to be colloidal. These are not, since they diffuse through the collodium film. Again, these intracellular poisons are probably of the nature of proteid compounds and thermo-labile. The poisonous substances here have been found to be thermo-stable. And, finally, if the antagonism is due to similar intracellular poisons, one would expect that the poison of the typhoid germs would be quite as antagonistic for itself as other intracellular poisons are for it. This is not true. The typhoid germ will grow on itself, to a somewhat limited extent, to be sure, but in striking contrast to the condition of affairs in the other cases.

THEORY OF ACID AND ALKALI PRODUCTION.

It has further been suggested that the antagonistic action is due to changes in the reaction of the culture medium. Sirotinin[19] suggested this, and claimed that by neutralizing the medium on which the organism was grown the inhibiting influence was destroyed. This explanation was rejected by Olitzky[3] in the study of Ps. fluorescens, and later by Horrocks,[7] who took gelatin slopes from which the growth of Ps. fluorescens had been removed, melted

*P. 34. Unfortunately the copies of the Local Government Board Report containing this have not been available to me, and I have been unable to determine on what experimental evidence Dr. Klein bases this explanation.

them up, and neutralized the gelatin without favoring the subsequent growth of B. typhosus.

In this work it has been found that all of the mixtures in which the collodion sac were used were strongly alkaline, varying from —0.7 to —1.2 (Fuller's scale); *i. e.*, they required from 0.7 to 1.2 c.c. $n/20$ HCl to neutralize 5 c.c. of the culture medium. That this alkalinity, as hydroxyl ions, would not in itself account for the death of B. typhosus seems certain, since it has been found repeatedly that the typhoid germ can grow luxuriantly where sodium hydrate (NaOH) is present so as to give a reaction of —1.5. All of the micro-organisms which have been found to antagonize B. typhosus are strong alkali producers, but in no case do they produce a greater quantity of alkali than that which corresponds to 15 c.c. of normal sodium hydrate per liter of medium, and, as stated above, this degree of alkalinity permits the ready growth of B. typhosus, so that it seems evident that it is not simply the alkalinity of the medium which produces the antagonism in the case studied here.

GENERAL CONCLUSIONS.

The most important of the conclusions which have been reached as the result of this study may be summarized as follows:

1. There is a marked antagonism exerted by mixed cultures of bacteria obtained from the soil and water on B. typhosus when the same are grown in broth and a collodion sac containing the typhoid germ is immersed therein.

2. This antagonism results in not merely checking the growth, but in actually killing the typhoid germs. In many cases the killing off amounts to extinction.

3. The death-rate of B. typhosus is more or less rapid, depending on the period of preliminary cultivation of the antibionts.

4. There is no evidence to show that the antagonistic substances exist ready-formed in the soil or water, but rather that the antagonism depends on the rapid development of the germs in the immediate presence of B. typhosus.

5. These antagonistic bacteria are widely distributed in nature, being present in various types of soils and waters.

6. An antagonism has been definitely associated with several different species of bacteria, viz., B. vulgatus, B. vulgaris, Ps. fluorescens, and Ps. putida. Of these the first-named is assigned this rôle for the first time; the second has been suspected of producing an antagonism; while the third and fourth have been associated with this phenomenon by previous observers.

7. Changes in the environment of these organisms, such as temperature, oxygen supply, reaction of medium, amount of dextrose, etc., seem to have little or no influence on the production of the antagonistic substances. In other words, whenever the environment is such that a good growth of the organisms occurs, the antagonistic substances are apparently always produced.

8. The energy with which the antagonistic substances act depends on the temperature. At 38° C. the action is very pronounced. At the temperature of the ice-chest (10–12°) the typhoid germ may grow in the by-products of the other germs, which at higher temperatures are quickly fatal.

9. The antagonistic substances are thermo-stable, being able to withstand a temperature of 120° C. for at least ten minutes.

10. The cause of the antagonism is not due, in the cases studied, to the exhaustion of the food supply, the action of proteolytic enzymes, specific poisons, or the production of hydroxyl ions simply.

11. This antagonistic action is not due to any peculiarity of a single typhoid culture, but is equally noticeable in at least three different strains.

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EXPLANATION OF PLATE XVIII.

FIG. 1.—Double-plate method of studying antagonism. The Petri dish is divided into two halves by means of a small glass tube which is sealed to the dish with collodion. In one half is poured sterile agar, and into the other half agar thickly seeded with the antibiont. After this has been incubated for some time, in this case forty-eight hours, streaks of the Bacillus typhosus are made across the surface of the entire plate. The photograph is taken forty-eight hours later. There is practically no growth of B. typhosus on the infected parts of the plates. *A* is Bacillus vulgatus; *B* is Bacillus vulgaris; *C* is Pseudomonas fluorescens; and *D* is Pseudomonas putida.

FIG. 2.—Agar-block method of illustrating antagonism. *A*, sterile bouillon showing typhoid streak, one month old; *B*, heated culture of Pseudomonas fluorescens, no growth, same age as *A*; *C*, sterile bouillon one week old growth; *D*, Bacillus vulgatus; *E*, Bacillus vulgaris; *F*, Pseudomonas fluorescens; *G* Pseudomonas putida, showing slight growth. Cultures a week old when not otherwise stated.

PLATE XVIII.

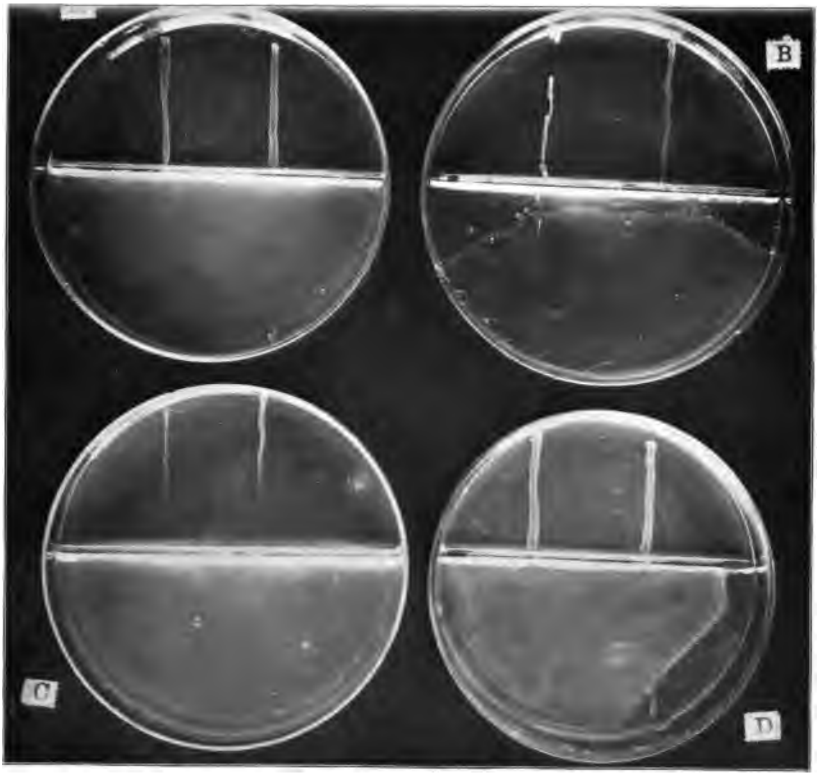

FIG. 1.